PECULIARITIES OF ABSORPTION SPECTROPHOTOMETRY OF BIOLOGICAL OBJECTS. II

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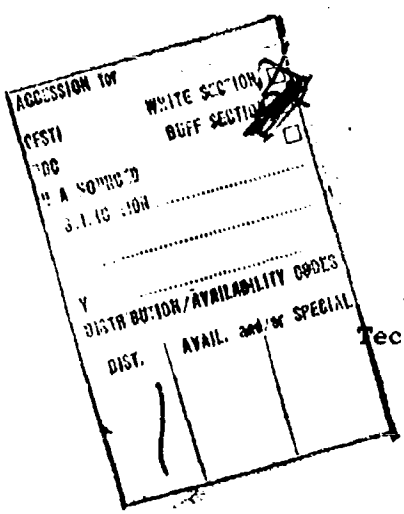

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1. Spectrophotometry of Dispersing Objects.

Until now it has been assumed that light, in passing through an absorptive medium, does not change the direction of its dissemination, that is, it is not scattered. However, practically all colored biological objects (leaves, tissues, retina of the eye, blood serum, etc.) diffuse light. This is connected with their heterogeneity in an optical respect -- with sharp changes in the refractive index, caused by the presence of various interfaces, minute particles, etc.

As was already said, scattering, first of all, influences the type of absorption spectrum and the absolute magnitude of the absorption coefficient, and, secondly, leads to certain peculiarities of a methodical nature.

First of all we will examine the methodical aspect of the problem.

A. Measuring the absorption spectrum with the help of a plane detector. First of all we will assume that a parallel pencil of light falls on the object, and beyond the object there is a stationary plane detector, just as in the majority of spectrophotometers which are usually employed. Determination of the absorption coefficient, just as earlier, should be reduced to a determination of the stream of light falling onto the object, and the stream of light coming out of the object, that is, not absorbed by it.

Measuring the flow of a parallel pencil of incoming light is no different from a similar measuring in the case of a scattering object and can be easily carried out with the help of a plane detector.

Something essentially new comes up when measuring the flow of outgoing (nonabsorbed) light. Due to scattering only part of the rays coming out of the object reach the detector (figure 1, a'). As was already noted the apparent absorption coefficient determined by this method is overestimated, since the true intensity of the outcoming light is greater than the measured value. In an extreme case the conditions for measuring may be such that the detector records only rays which pass through the object without changing the direction of dissemination (for example, when the detector is located at a great distance from the object).

When a parallel pencil of rays falls on a thin absorbing and scattering layer of substance, a weakening in the flow of light which reaches the detector is caused not only by the actual absorption, but also by scattering, and may be described by the formula

$$dI = -(\varkappa + \sigma) I\, dx,$$

where $\varkappa$, just as before, designates the absorption index; σ is called the scattering index and $\varepsilon = \varkappa + \sigma$ -- the reduction coefficient (sometimes extinction). This formula may be used for determining the flow of only those rays which pass through the object without essentially changing their direction, but it cannot be used for calculating the flow of all the rays coming out of the object, that is, for determining the overall passage coefficient (and, consequently, also the absorption coefficient).

As pointed out above, the main error in the method of measuring with the help of a stationary plane detector is that not all the nonabsorbed rays are captured by the detector. With such an erroneous method, it is possible to obtain large values of the apparent absorption coefficient for scattering objects which do not contain pigments at all and therefore, in actuality, do not absorb light. For example, in albino leaves of plants the true absorption coefficient is usually less than 10%, while at the same time when measuring in parallel rays the apparent coefficient may reach 90% and higher, depending on the geometric conditions of the test.

From what has been said it is clear that with the help of ordinary spectrophotometers, for example the Bekmen SF4 or SF5 spectrophotometers, it is impossible to obtain correct absolute values of the absorption coefficients of scattering objects.

From figure 1 it is also easy to understand why the value of the apparent (that is, measured) absorption coefficient depends on the geometric conditions of the test. The most important of these conditions is the distance between the object and the detector. The closer the detector is to the object then the greater will be the number of rays which are captured by it, and then the closer to true value will be the current of outcoming light (compare positions a' and b' in figure 1).

In exactly the same manner, the greater the cross section dimension (diameter) of the detector then the closer to true value will be the measured absorption coefficient.

Nevertheless, even in the event the object is flat and is moved close to a plane detector which has as great a diameter as desired, all the same only those rays will be recorded which come out from one side of the object; rays coming out from the side of the object which is turned toward the source of the rays (reflected rays) will not be recorded even in this case. In this manner, by means of moving the object closer to the detector it is possible to obtain apparent values for the absorption coefficient which come closer to the true value, but all the same a great deal of this /value/

if left out. It can be said that the greater the solid angle at which the detector is seen from the object, the closer to the correct value will be the measured absorption coefficient obtained. Apparently a correct value is obtained when the detector completely surrounds the object, that is, when the stated solid angle equals 4π and all the outcoming rays are captured.

Noncalculation of all the rays coming out of the object sometimes leads to very instructive phenomena.

We will examine several particularly interesting cases.

The spectrum of absorption of a solution of oxyhemoglobin has a very strong band (gamma band) in the violet band of the spectrum ($\sim$ 410 millimicrons) which disappears during measurements with a suspension of erythrocytes /1/. It was demonstrated /2/ that this disappearance is observed when primarily rays are being recorded which have passed through the suspension without scattering. If the geometry of the measurements is such that the scattered rays are also recorded, for example if the detector is moved right up to the cuvette with the suspension, then the gamma band makes its appearance. In this manner the apparent value of the absorption band essentially depends on the method of recording the scattered rays. Possible explanations of this phenomenon are given in the works /3/ and /4/.

Here is another example. Measurements with suspensions of the green algae Chlorella showed that the position of the absorption band changes depending on the geometric conditions of measuring. Thus when recording mainly nondeflected rays the maximum of the absorption band turns out to be shifted to the long-wave side in comparison with the position of the maximum when recording scattered rays /5/ (figure 1). A probable explanation of this effect is that light with a wave length corresponding to the long-wave slope of the absorption band scatters more strongly than light with a shorter wave length (phenomenon of selective scattering) /16/. As a result, when recording nondeflected (passing) rays the absorption of strongly scattering, that is, long-wave, rays will turn out to be great, and the maximum of absorption shifts to their side. If the detector catches the scattered rays also, then the apparent absorption coefficient of these more strongly scattering, that is long-wave, rays will be less.

Zhidkova's tests with colored powders /7/ have a close relationship to the effect under discussion. It was shown that the spectral curve of the coefficient of reflection of a strongly scattering, but relatively weakly colored object in general has the same form as the spectrum of transmission through absorbing substance in mass (in a homogeneous condition). However, in some cases the curves of reflection and transmission differed strongly and, in particular, a significant shift of the maximum took place. This shift (just as the coincidence of the maximums in the event of a strongly dispersed but weakly absorbing substance) is easily explained by the theory which allows for the dependency of the relative index of refraction of the colored substance and the medium on the wavelength /8/. The theory shows that the shifting of the maximum should take place when the dispersion

curves of the absorbing substance and the dispersion curve of the medium are strongly oblique to each other (they intersect at a large angle). Such an intersection takes place in particular within the limits of the absorption band of a strongly absorbing substance.

An absolutely paradoxical result may be obtained if the object has great length and the detector primarily records light which is running along a specific direction, but corresponding with the direction of the incident light. As an example we will take the case which is depicted in figure 2. On account of the scattering the distribution of light based on direction changes sharply in the object and the intensity of light reaching the detector, which is recording light at a certain angle to the initial direction of light, at first will increase with the thickness of the absorbing layer. It is formally arrived at that the "coefficient of transmission" is greater than unity or the coefficient of absorption is negative. Actually, of course, the total amount of light, passing through any plane in an object, perpendicularly to the initial direction of the rays, decreases with depth but the intensity of the scattered light may increase according to the degree of penetration into the depth of the medium. With sufficiently large depths the intensity will decrease also along this direction. This will take place when absorption prevails over the effect of the directional redistribution of light.

In this manner a curve, depicting the dependency of the intensity of passing light on the thickness of the layer, may show a maximum. Figure 2 shows the curves which are obtained when the detector is immersed at a various depth in a medium made up of diluted milk; the detector was sensitive to rays at 6--20° to the direction of the incident rays /9/.

Similar effects of "focusing" are not specific for light rays, and are also observed for gamma rays /10/ and electrons /11/. This is explained by the fact that in spite of the existing differences in the mechanisms of scattering and absorption of various types of rays, the laws of their propagation through scattering and absorbing media remain the same.

It is clear from what has been said that to measure the absorption coefficient of a scattering medium with the help of a directional detector would imply an even greater worsening of that error which is obtained when working with a plane detector, which is directional to a lesser degree and therefore records a large share of nonabsorbed rays.

It is necessary to again note that the majority of plane receivers (photocells, thermopiles, pyranometers) are selectively sensitive for direction, that is, do not have the same liklihood to record rays coming in at various angles (do not possess a cosine nature), and to a certain degree this effect may also influence the measurement.

Distortion of the absorption spectrum may be eliminated to a known degree with the help of the opal glass method, first proposed by Pokrovskiy

/12/. If diffuse scattering glass is placed between the scattering object and the detector, then those rays which are scattered at various angles will enter the detector (but at no greater an angle than 90°). Consequently, the effect of the uneven scattering of rays of various wavelengths in the object is eliminated to a significant degree by this method and a shift of the absorption maximum cannot be observed. Besides this, more likely absolute values of the absorption coefficient are obtained, since the measuring of the incoming light is also carried out with glass and consequently the weakening of the light due to scattering in the object is partially taken into account. However this method does not yield either a correct absolute value of the absorption coefficient or the correct form of the absorption spectrum /13/, but it possesses the quality that the position of the absorption band, as was already said, may be obtained correctly /6/.

If the mission is to determine the optical properties of the absorbing substance as such, then when working with suspensions of cells it is possible to eliminate the influence of scattering with the help of the above mentioned method of clarification, based on the selection of a medium with a refraction index close to the refraction index of the cells /14/.

In summing up, it can be said that for measuring the absorption coefficient of a nonscattering object onto which a parallel beam of light is falling, it is sufficient to make two readings with the help of a plane detector; to measure the incident and transmitted beams (just as everywhere, we consider introducing, by the appropriate method, the small correction for reflected light). To determine the true absorption coefficient of a scattering object, based on what was presented above, is impossible by the stated method. In the next section methods will be examined for determining the true absoprtion coefficient.

B. Methods for measuring the absolute value of the absorption coefficient of a scattering object. A detector which records only part of the outcoming light (plane detector) may be used all the same in principle for determining the total stream of outcoming light. For this it is sufficient to sum up the streams, measured at all possible angles, by moving the detector along the surface of a sphere, in the center of which the object under investigation is found (figure 3, a).

A similar goniometric unit was used, as an example, for measuring the coefficients of reflection and passage of the skin of man /15/ and the leaves of plants /16/.

The main deficiency of the goniometric method is the amount of labor required for the measurements. Determination of the coefficient of passage usually requires many minutes and sometimes even hours. The measuring is essentially simplified if the intensity of light is symmetrically distributed relative to the direction of the incoming light; then for measuring the absorption coefficient it is enough to measure the distribution of the outcoming light in any one plane, passing through the direction of entering ("indicatrix of scattering"), and from here to calculate the total intensity of light coming out of the object being investigated.

A merit of the goniometric method is the feasibility of obtaining a picture of the distribution of intensity of scattered light along various angles, which may prove to be desirable in certain investigations.

The variety in the goniometric method is that a large number of similar detectors are distributed on the inner surface of a sphere which is surrounding the object (figure 3,b). By this method it is possible to obtain the value of the stream of outcoming (scattered) light by means of summing up the readings of all the detectors. Here it is necessary to introduce a correction for light which does not reach the detectors. Based on the readings of each of the detectors separately, it is possible to make an approximate determination of the spatial distribution of the stream of scattered light. The light, reaching the detectors and the nonsensitive surface of the sphere, should be completely absorbed, otherwise the deflected light may distort the true picture of scattering. In practice it is possible to dispose the detectors on the facets of a polyhedron, for example, a dodecahedron (20 facets) /17/.

The condition for using the described "detector polyhedron" is the absence of a sharp dependency of the intensity of the scattered light on the angle, since it turns out that at certain angles of a particularly intense scattering the light does not reach a detector and the stream of scattered light turns out to be too low. This comment is also in regards to the above described goniometric method, if the angles are changed discretely.

Instead of determining the total flux of light by integrating it based on all the angles with the help of one detector (goniometric method) or with the help of many detectors (polyhedron method), it is possible to determine the total flux of light, scattered at all angles, with the help of the so-called method of integrating spheres. At the present time this method has been used extensively. The object is placed in the center of a sphere of sufficiently large dimensions. In the sphere a small opening is made through which the incoming light passes, (figure 3, c) and a second opening over which the light detector is attached. The inner surface of the sphere is covered with a layer of aneasily scattering but weakly absorbing substance, for example MgO or $BaSO_4$. The idea of the method is that the light coming out of the object, being scattered numerous times, creates a certain average illumination within the sphere, and this illumination is proportional to the total stream of light which is not absorbed by the object /18/. In this manner it is as if the sphere sums up ("integrates") and more precisely averages out the light going along all directions from the object. The reading of the detector will be proportional to the average illumination under the condition that light does not reach the detector which is coming directly from the object, but only the rays which have been reflected numerous times. For ensuring this condition etched or opal glass is placed within the sphere between the detector and the sample. This glass scatters the light coming directly from the object to the detector. Besides this the object itself should absorb the rays which have been reflected numerous times to the minimum degree, and therefore the dimensions of the sphere should be great in comparison with the size of the object. In practice it is sufficient that the diameter be ten times greater than the cross-sectional dimension of the object. In exactly the same way

the area of the incoming and outgoing (at the detector) openings should make up an insignificant share of the entire area of the sphere. Several problems of working with the integrating sphere are presented in /19/.

Two readings are made for determining the absorption coefficient: The first in the absence of the object, or, if the sample is a liquid, in the presence of a cuvette with a nonabsorbing liquid. The reading of the device in this case corresponds to the stream of incoming light I_0. It would be best of all to make a control (nonabsorbing) sample from material which scatters light approximately the same as the object under investigation, since in practice the average illumination may nevertheless depend somewhat on the angular distribution of light initially reaching the inner wall of the sphere.

The second reading in the presence of the object being investigated corresponds to the stream of light coming out of the investigated object in all directions (I_t). The stream of absorbed light will be $I_0 - I_t$, and consequently the absorption coefficient $A = (I_0 - I_t)/I_0 = 1 - I_t/I_0$. An example of the application of this method is the measurement of the spectra of absorption of plant leaves /20/.

If the investigated object is a thin membrane (for example, a leaf), then the apparatus can be simplified. Instead of introducing the object into the sphere it is possible to determine separately the stream of passing light (I_{pr}) and the stream of reflected light (I_{otr}). For determining I_{pr} it is necessary to place the sample before the incoming opening of the sphere (position 1 on figure 3, c); for determining the I_{otr} the object is placed behind the opening, cut out on the opposite end of the diameter, passing through the inlet (front) opening (position 2). When measuring I_{pr} the second (rear) aperture is closed with a plate which is covered with the same reflecting material as the inner surface of the sphere. The absorption coefficient will equal $A = [I_0 - (I_{pr} + I_{otr})] / I_0$. The advantage of this method is that it is possible to determine individually the "passage coefficient" $T = I_{pr}/I_0$ and the "reflecting coefficient" $R = I_{otr}/I_0$. Obviously $A = 1 - (T + R)$.

As already stated we use this variation of the method of the integrating sphere only for thin objects; besides this, the walls of the sphere should be sufficiently thin. If the stated conditions are not fulfilled, then part of the passing or reflected light will be absorbed by the walls of the sphere. This variety of the integrating sphere method has found wide application in spectrophotometry /21/ and differential spectrophotometry of plant leaves /22/.

In the recording spectrophotometers of the SF 10 type and the so-called spherical photometer (ShF), spheres with two apertures are used according to the method described.

C. The influence of scattering on the true absorption spectrum. In the previous sections the methodical peculiarities of measuring the absorption coefficient of scattering objects were reviewed. Mainly these peculiarities are conditioned by the necessity to take into consideration rays which are coming out of an object in various directions.

Now we will raise the following two problems: 1) how does scattering influence the true (that is, correctly measured) value of the absorption coefficient, and 2) how does the form of the true absorption spectrum depend on scattering.

1. Due to scattering the length of the path and the direction of a light ray in a turbid object will be different in a similar but nonscattering object. As a result of scattering, the path length of individual light rays may be either increased (for example, rays 2 and 3, figure 4) or decreased (rays 1 and 4).

If the change of the path length increases the probability of an encounter between the light rays and the molecules of the absorbing substance, then absorption, apparently, is increased. However, scattering may decrease this probability (for example, due to reverse scattering) and, consequently, lead to a decrease of absorption. Therefore the presence of scattering does not always lead to an increase in the absorption coefficient.

Which of these two cases takes place depends on a quite complex interweaving of various factors, and mainly on the relationship between the absorptive and scattering capability of the substance of the object, on the mutual distribution of the absorbing and nonabsorbing substances, and the nature of light incidence.

For an illustration of this we will examine an object in which the pigment is disposed in the form of a thin concentrated layer, and the remaining part consists of a colorless (that is, nonabsorbing) substance. We will assume initially that the colorless layer does not scatter light and permits it to hit perpendicularly on the object (figure 4, b). It is evident in this case that the absorption coefficient of the object being examined will not depend on how the light is moving -- from top to bottom or bottom to top.

If the nonabsorbing substance scatters light strongly, then the absorption coefficient may not differ essentially from the absorption coefficient of the nonscattering object, if the rays hit below, that is, directly on the pigment layer; but it may be less if the rays pass initially through the colorless scattering layer (figure 4, a), since part of the rays will leave the object without encountering the pigments. In the last case we have an example of when scattering leads to a decrease of the absorption coefficient, in spite of the fact that the average path length of the light rays in the sample was increased.

Not only does the distribution of pigment have significant meaning, but also its absorption index and concentration. Actually we will visualize that the pigment is uniformly distributed in the sample. If the optical density is such that in the absence of scattering practically all the light would be absorbed, then scattering will lead only to a certain decrease in the absorption coefficient, due to the reverse scattering ("reflection") of rays. If a pigment with a small optical density is uniformly distributed in the object, then the absorption coefficient may be increased significatnly due to scattering.

The increase of absorption in a turbid medium as a result of the elongation of the optical path of light may be used for the identification of pigments in highly diluted solutions. Thus the addition of the powder of $CaCO_3$ or Al_2O_3 led to a strong (by many times) increase of absorption and thereby made it possible to detect the characteristic, but weak, absorption bands of phytochrome /23/.

The sum conclusion, which can be made on the basis of the above cited examples, is that, depending on the size of the sample, concentration, absorption capability (absorption index) and distribution of pigment, and also the direction of light incidence, scattering may lead to either a decrease or an increase in the absorption coefficient.

2. It is not difficult to understand that scattering should also change the appearance of the absorption spectrum, that is, lead to a nonproportional alteration of the absorption coefficient at various wavelengths. For example, let the conditions be such that the scattering leads to an increase of the absorption coefficient (that is, there is an increase in the path of the light ray in the absorbing area within the sample) and let light with a wavelength of λ_1, be absorbed very strongly and light with λ_2 be absorbed weakly. Then an increase of the effective path of the rays within the sample has little influence on the absorption coefficient at , and may strongly change the absorption coefficient at λ_2. As a result the absorption spectrum will "wear smooth." For an illustration of this phenomenon, figure 5 presents the absorption spectrum of a leaf, infiltrated with water (the water creates optically more uniform conditions within the leaf and therefore decreases scattering), and the absorption spectrum of the same leaf in a normal condition (curve a) /24/. This example again shows the nonjustification of introducing the concept "effective leaf."

Theoretically these problems were examined by Rozenberg, who was able to obtain simple formulae for calculating the influence of scattering on the form of the absorption curve /25/.

It could have been thought that in spite of the change in the appearance (leveling) of the absorption spectrum, caused by scattering, the appearance of the spectrum of optical density (that is, curve $D(\lambda) = -\ln(1-A)$) remains as before. But actually, during the leveling of the absorption spectrum a nonproportional change takes place in the ordinates of the spectrum of optical density.

The Lambert-Buger Law, strictly speaking, loses its significance in the case of non-uniform media. The essence of this law is that a layer of the same thickness absorbs the same share of fraction of light reaching it. In the case of a non-uniform medium, when layers of the same thickness may differ strongly physically and chemically, it is very apparent that the intensity of light may decrease irregularly. What has been said does not exclude the possibility that in some cases (for example, a suspension of uniformly distributed minute pigmented objects) a decrease in the transmitted

beam will lessen exponentially with thickness, in any case in a certain interval of a change in thickness.

2. Differential (Difference) Spectrophotometry

With the help of spectrophotometry it is possible to study the kinetics of various processes in which colored components take part. For example, information concerning the participation of cytochromes in the transmission circuit of electrons during respiration was obtained by means of tracing the change of cytochromes through their absorption spectrum [26]. However, stained components under investigation are usually found in a modified condition for a very small interval of time, and therefore, the accompanying spectral changes turn out to be small, on the order of or less than one percent. It is not possible to measure such small changes with the help of ordinary spectrophotometers. Based on this reason, instead of measuring the absorption spectrum before the process and during its course, and comparing these spectra, it is expedient to directly measure the difference between both spectra. Then knowing the spectrum of the initial pigment, it is possible on the basis of the difference or "differential" spectrum to determine the form of the spectrum of the modified substance and, consequently, to make a judgement concerning the nature of change of the original pigment during the process being investigated.

Methodically, measuring of the difference spectrum amounts to the following: The transmission coefficient of the initial pigment at a given wavelength is conditionally taken as zero, that is, the passage coefficient of the modified pigment is reckoned based on the relationship to the stated coefficient.

For a photoelectric spectrophotometer this can be done by compensating the current from the passing light by the current of the opposite direction. Various methods of compensation exist [27], but we will not examine them here. If at the present time the pigment in the object being investigated is changing under the influence of this or that factor, then the device will indicate the change in the passage coefficient in relation to the initial one. Curve 1 in figure 6 depicts the spectrum of reduced cytochrome c. Following oxidation the spectrum takes on the form depicted by curve 2. The differential spectrum is determined by the difference between curves 2 and 1, and is depicted by curve 3. The spectral transition of cytochrome c from a reduced to an oxidized condition is manifested in the emergence in the difference spectrum of a strong minimum at 420 millimicrons and maximum at 400 millimicrons, and also two small minimums at 520 and 550 millimicrons. Consequently, the simultaneous emergence of similar characteristic peaks in a living object may be viewed as an indication of the oxidation of cytochrome c.

The expediency of the direct measurement of the difference spectrum is seen from the following example. We will assume that the passage coefficient T = 50% and following recording of the passing light the device showed, as an example, 50 units. If the passage coefficient of the modified pigment is T = 50.2%, that is, the passage coefficient has changed by 0.2%,

then the difference in the readings on the indicator will be all told 0.2 units. In the differential method of measuring, when the initial current has been compensated for, it is possible to strongly increase the sensitivity of the device so that in place of 0.2 the device would show, as an example, 20 units. Without compensation of the initial current, apparently, it is impossible to achieve a point where a change of T by 0.2% would produce a deflection by 20 units, since in place of 50 units the device would initially indicate 5,000 units. From this example it is apparent that the differential method of measurement makes it possible to significantly increase the sensitivity of the device to small changes in the passage coefficient.

At the present time, with the help of differential spectrophotometers it is possible to measure relative changes of the passage coefficients, $\Delta T/T$, all told of 0.01 and even 0.001% /28/.

It must be kept in mind that in optically non-uniform objects, such as biological objects, the nature of spectral changes may be determined not only by the chemical changes of the pigment, but also by changes in its physical state and in the state of the medium. Since in differential spectrophotometry very small changes of the absorption coefficient are recorded, then the role of purely physical factors may be important and the interpretation of the results may be made much more difficult.

We will examine the following examples. Figure 7 (I) depicts the absorption spectrum of leaf a and the absorption spectrum of a suspension of crushed chloroplasts isolated from it, b, that is, after its homoginization /24/. The difference spectrum a--b is depicted by the curve (II). As can be seen, the change of the geometrical distribution of the pigments (increase of non-uniformity) led to the appearance of a complex differential spectrum of a leaf or a suspension of green algae, emerging following irradiation of the plant with light /29/ (figure 7, III). Thus is it completely possible that the differential spectrum of plant leaves, emerging following the irradiation of the latter with light is conditioned, in any case partially, by a certain change in the distribution of pigments in the leaf under the influence of exciting light.

In certain cases the emergence of the characteristic differential spectrum may be partially conditioned also by the peculiarities of the apparatus used. This may be illustrated by the following example. As was already pointed out, due to the selective scattering the apparent absorption spectrum of the scattering object, measured with the help of a plane or in general a directional detector, depends on the angle of scattering of the recorded rays. In all the differential spectrophotometers used at the present, passing rays are recorded, that is, rays constituting an angle $< 90^{\circ}$ with the direction of the incident rays. Consequently, if the influence on the object changes its scattering ability, for example, increasing the degree of dispersion of the protoplasm, then the detector will record the weakly deflected rays to a greater extent. The spectrum of these rays, due to the phenomenon of selective scattering which we spoke about previously, differs from the spectrum of

the strongly deflected rays. Thus under the influence of the stated effect, the form of the apparent absorption spectrum is changed, that is, a differential spectrum appears. If, for example, the irradiation of a suspension of Chlorella increases its scattering ability, then on the basis of curves a and b in figure 1, it is possible to expect the appearance of a differential spectrum of the optical density shown in figure 7, IV. Attention should be paid to the general similarity of curves III and IV in figure 7.

Everything that has been presented shows that the interpretation of the differential spectra of biological objects is a complex problem and can hardly be achieved by means of a simple comparison with the differential spectra of known colored substances in homogeneous media. It seems that a preliminary condition for the successful interpretation of differential spectra is a comprehensive investigation of the influence of various factors on the physical state of an optical medium and the appearance of possible spectral effects as a result of these physical changes of the medium.

3. Derivative Spectrophotometry

In recent years in biology they have started to apply the so-called derivative spectrophotometry. The idea of this method is that the determination is made not of the spectrum of absorption $A(\lambda)$, but of the curve of dependency on λ of the derivative of the absorption coefficient (or optical density) with respect to wavelength (that is $\frac{dA}{d\lambda}$). In principle such a curve can be obtained if the absorption spectrum based on the wavelength is differentiated. However, it is more accurate to directly measure such spectra, and for this purpose special spectrophotometers have already been produced, which at once trace out the differentiated (do not confuse this with differential, that is, difference, spectrum) spectrum of absorption based on λ [30].

The advantages of derivative spectrophotometry are that with its help, first of all, it is possible to more accurately determine the position of the absorption maximums, and secondly, it makes it possible to reveal the presence of absorption maximums which in the ordinary spectrum of absorption are completely hidden. The value of similar measurements is determined by the fact that based on the position of the maximums it is often possible to judge the condition of the pigments (degree of aggregation, bond with other compounds, etc.).

Since the derivative at the maximum equals zero, then the position of the maximum in the derived (differentiated) spectrum is established as the point of intersection of this curve with the x-axis, that is, axis λ . As an example, in figure 8 we present a sector of the spectrum of optical density and the differentiated (derived) spectrum of chlorophyll a in ether. For an illustration of the possibilities of derived spectrophotometry in relation to the exposure of maximums which do not appear in the ordinary absorption spectrum, we will take a hypothetical case when the absorption band consists actually of two bands with a similar position of peaks, but

having a different width and height (figure 9,a). Based on the resulting spectrum of passage (curve b) it is difficult to guess of the presence of two peaks. The complex structure of the differentiated spectrum of passage (curve c) clearly indicates the existence of several absorbing components /31/.

For our presentation it is important to note the following. Errors when working with scattering objects which are caused by an incorrect calculation of the scattered rays, are not decreased when using derived spectrophotometers in place of ordinary devices. In other words, when determining the position of the absorption maximums of scattering objects with the help of derived spectrophotometry, it is necessary to observe the same precautions which were reviewed above. This is also true in regards to difference spectrophotometry.

It can be said that for an accurate determination of the absolute value of the absorption coefficient, and also the position and number of absorption bands, the most important condition is that a correct calculation of all the rays not absorbed by the object be made, regardless of which method of spectrophotometry is used -- the ordinary, the difference, or derived varieties of it.

Conclusions

In an optical respect biological objects are distinguished by their heterogeneity.

An uneven distribution of absorbing substance in an object always leads to a decrease of the absorption coefficient in comparison with the coefficient of the corresponding hemogeneous object. The absorption coefficient decreases most noticeably in the area of strong absorption. As a result a "leveling" of the absorption spectrum takes place. Besides this, there occurs (in any case for a suspension of colored particles) also a leveling of the spectrum of optical density, formally determined as $D = -\ln(1-A)$.

The influence of light scattering on the absorption spectrum depends on the scattering and absorbing capabilities of the substance of the object, on their distribution in the object, and on the direction of incident light.

If the scattering and absorption are not very great (so that a significant part of the rays penetrates into the depth of the object), then scattering most rapidly of all will lead to an increase of the absorption coefficient. With a very strong scattering, when a noticeable part of the rays scatter reversely and therefore do not cover a significant path in the object, and also during a strong absorption when the lengthening of the optical path of the ray within the object hardly influences the absorption coefficient, scattering may lead to a certain lowering of the absorption coefficient. Thus, under the influence of scattering a leveling of the absorption spectrum may also take place.

A methodically correct measurement of the absorption coefficient of a scattering object amounts to the correct calculation of all the nonabsorbed

("outcoming") rays. The most suitable method for obtaining correct absolute values for coefficients of absorption is the method of the integrating sphere. In the case of an absence of isolated directions of scattering the detector polyhydron method is suitable. For determining the position of the maximums on the absorption curve in certain cases it is possible to use ordinary spectrophotometers which utilize plane detectors. In the common case, however, the form of the absorption curve, and in particular the position of the maximums, may be distinguished from those which are obtained when calculating all the outcoming rays. Therefore, if changes are being studied in the position of the maximums of absorption, conditioned by these or those factors, then when using the stated method it must be ascertained that the observed shifts are caused mainly by a change in the absorption spectrum of the pigment and not by a change of scattering.

Somewhat more reliable results may be obtained when using ordinary spectrophotometers by means of using diffusely scattering (opal) glass. However, even when applying this method the position of the bands is not always obtained correctly. If small distortions in the form of the absorption curve do not have any significance in a specific investigation, then any of the mentioned methods may be recommended.

This article did not examine the problem of determining the absorption index of the substance of an object based on the measured absorption spectrum of the object. Essentially only a resolution of this mission will make it possible to obtain accurate data concerning the nature of substances in an object, their state, their changes, etc. The theoretical aspect of this problem, though it has been developed quite intensively in recent years, is apparently still far from completion /32/. Nevertheless, it is without a doubt that already at the present time the theory is capable of rendering great assistance to biological investigations. A necessary condition for this is the closer tie between physicists working in the area of optics of scattering media and biologists.

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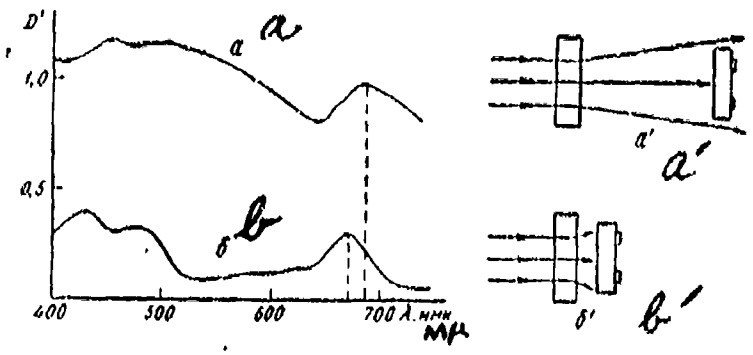

Figure 1. Dependency of the position of the maximum and the apparent value of the optical density of a suspension of Chlorella algae on the geometric conditions of the measurings.

Angle of abscissa -- length of wave, millimicrons; angle of ordinate -- optical density.

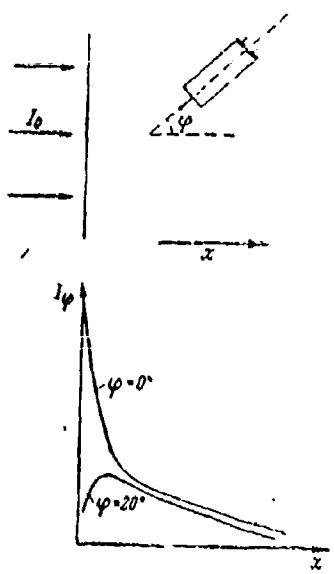

Figure 2. Dependency of the form of the light intensity curve in a turbid medium on the angle of distribution of the measured light.

x -- depth of detector in the medium; $I\varphi$ -- intensity of light, running at the angle φ to the falling light. Upper diagram -- arrangement for measuring.

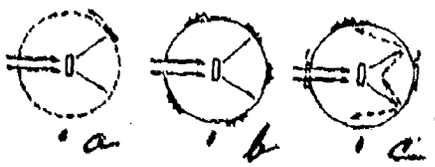

Figure 3. Various methods for determining the absolute value of the absorption coefficient with the help of a plane detector.
a - goniometric method; b - detector polyhedron method; c - Ulbricht sphere method.

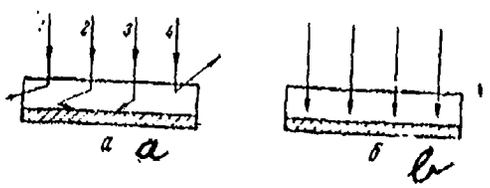

Figure 4.

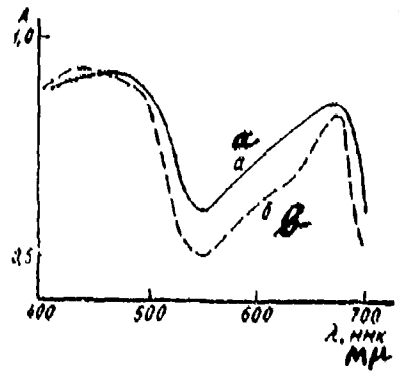

Figure 5. a -- spectrum of absorption of a leaf; b -- absorption spectrum of the same leaf following infiltration with water.

Angle of abscissae -- wavelength millimicrons; angle of ordinates -- absorption coefficient.

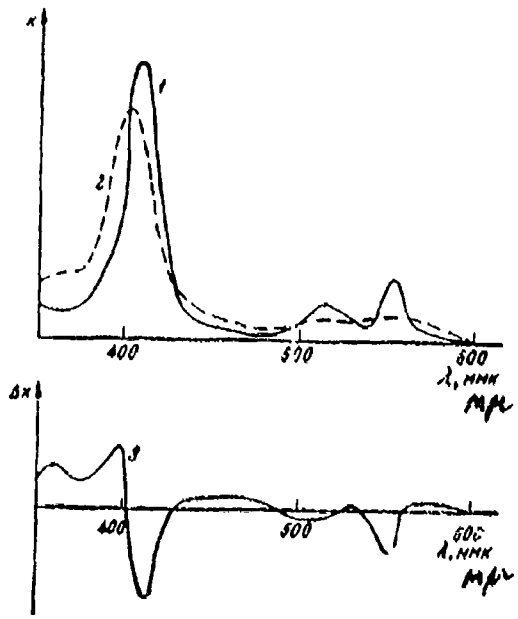

Figure 6. 1 -- spectrum of the absorption index of reduced cytochrome c; 2 -- spectrum of oxidized cytochrome c; 3 -- differential spectrum, obtained during the oxidation of cytochrome c.

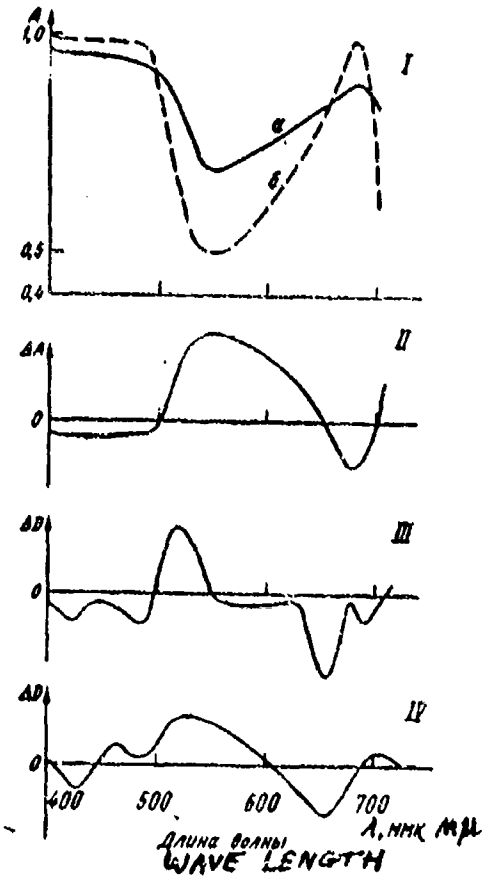

Figure 7. Diagram I: a -- absorption spectrum of a leaf; b -- absorption spectrum of a crushed and homogenized leaf.
Diagram II: difference spectrum (a--b).
Diagram III: differential spectrum of the optical density of a leaf or a suspension of green algae following irradiation of the plant with light.
Diagram IV: differential spectrum of the optical density of a leaf or suspension, anticipated as a result of a change of the scattering ability of the suspension of algae.

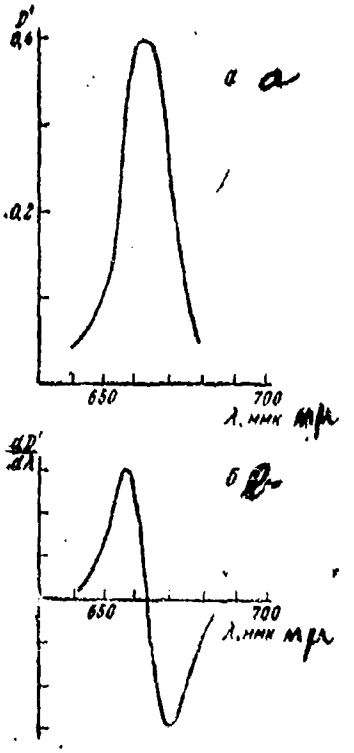

Figure 8 (a--b). a -- sector of the optical density spectrum of chlorophyll a in ether; b -- derived (differentiated) spectrum.

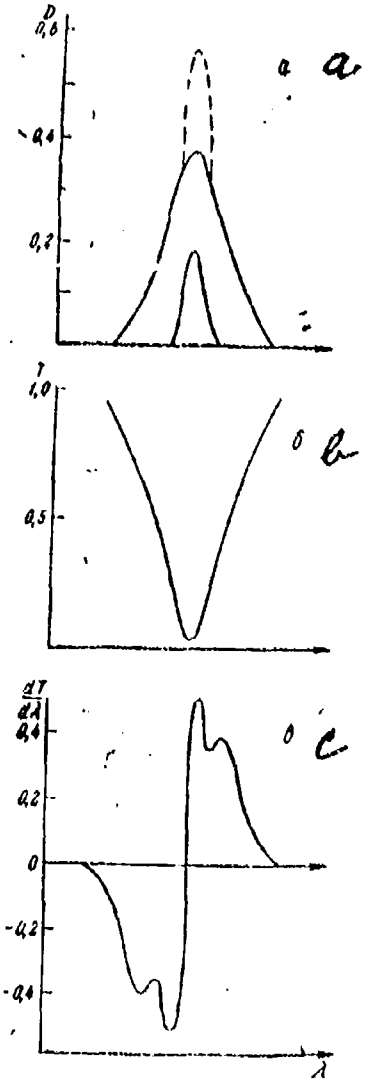

Figure 9 (a--c). a -- hypothetical spectrum of optical density, consisting of two bands with coinciding positions of maximums, but the width is different; b -- total spectrum of passage, calculated on the basis of the summary curve a; c -- derived (differentiated) spectrum of passage.

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Abstract

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This translation was prepared from an article originally published as follows: Marha, K. Biologicke ucinky elektromagnetickych vln o vysoke frekvenci. Pracovni lekarstvi, v. 15, no. 9, 1963, 387-393. Physicists, chemists, biologists, and doctors are all interested in finding out whether electromagnetic waves cause chemical and biological changes similar to those brought about by ionic radiation. Previous experimental work on the influence of high-frequency electromagnetic waves on organic and inorganic matter (begun at the end of the last century) is briefly covered. In this report the author discusses the influence of high-frequency electromagnetic waves on living matter, especially in the field of microwaves. He deals with penetration of high-frequency energy into the organism, electromagnetic induction of the body, and the existence of the non-thermal effect of electromagnetic waves. Symptoms of damage are listed and methods of protection discussed. The original article included 29 references. Four graphs are included at the end of the report.

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